The use of oxygen evolution to assess the short-term effects of toxicants on algal photosynthetic rates

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Abstract

O_2-production using either *Selenastrum capricornutum* or *Chlorella vulgaris* as indicator organisms to assess the presence or not of toxic compounds, was measured in a small volume oxygen chamber. These measurements were done at predetermined I_k irradiancies. At EC_{50} and EC_{90} levels, the response of *S. capricornutum* and *C. vulgaris* to atrazine toxicity was opposite to the response as determined at the EC_{10} level. *Chlorella vulgaris* is more sensitive than *S. capricornutum* to high atrazine concentrations, but *S. capricornutum* is more sensitive than *C. vulgaris* at the EC_{10} level. It was shown that the heavy metals Hg, Cd and Cu and the herbicide, atrazine, influenced the photosynthetic rates but the organophosphate, gusathion, had no effect. The oxygen evolution assay may be useful as a rapid preliminary screening method for the presence or absence of toxic substances.

Introduction

Concern over environmental perturbations has resulted in the search for methods of evaluating the effects of introducing substances to aquatic systems that may disrupt the equilibrium of those systems. The response of organisms to pollutants (excessive nutrients or toxic substances) is central to the disruption of equilibria and it is because of this that bioassays are used to evaluate the effects of pollutants.

In algal suspensions, changes in the dissolved oxygen concentration could be brought about either by photosynthetic evolution or respiratory depletion. From these changes two completely different metabolic pathways could be used in toxicity studies, i.e. photosynthesis or respiration. Hostetter (1976) developed a rapid bioassay sensitive to nutrients and the presence of toxic substances using a Clark-type oxygen electrode and a reaction chamber containing the water to be tested and the test organism. The rate of net photosynthesis as oxygen production was determined with this method. A prerequisite of such a method is that it is essential to reproduce the experimental conditions. Reproducing experimental conditions implies precise control of environmental conditions such as temperature and the light field, together with a rapid, reproducible, assessment of O_2-evolution. Clark-type O_2-electrodes offer advantages over other methods in that they allow for continuous measurements of oxygen production or consumption (Dubinsky et al., 1987). Net photosynthesis could be inhibited by toxic concentrations of substances.

In this study, results are presented where the oxygen chamber, as described by Dubinsky et al. (1987), was used to measure the possible effects of selected toxic substances namely; copper, cadmium, mercury, atrazine, gusathion and phenol (Table 1) on net photosynthetic rates as determined from oxygen liberation. The oxygen evolution assay may be useful as a rapid preliminary screening bioassay for determining which chemicals should undergo further testing.

TABLE 1
TOXIC COMPOUNDS AND THEIR CONCENTRATIONS USED IN THE TESTS

Compound	Concentration ($mg \cdot \ell^{-1}$)
Cadmium	0.005, 0.01, 0.05, 0.1, 0.25 and 0.5
Copper	0.01, 0.02, 0.2, 0.5, 1 and 2
Mercury	0.005, 0.01, 0.02, 0.05, 0.1 and 0.2
Atrazine (an organochloride)	0.005, 0.05, 0.5, 1, 5 and 10
Gusathion or Azinphos-methyl (an organophosphate)	0.00001, 0.0001, 0.001, 0.01, 0.1 and 1
Phenol	0.01, 0.1, 1, 10, 100 and 200

Material and methods

Unialgal cultures of *Selenastrum capricornutum* (CCAP 278/4) and *Chlorella vulgaris* (CCAP 211/12) were obtained from the Culture Collection of Algae and Protozoa (CCAP), Natural Environment Research Council, Cambridge, UK (now at the Institute of Freshwater Ecology, Far Sawrey, near Ambleside, UK). The reason for selecting these algae was that they maintain unicellularity throughout their life cycles which makes them suitable for cell counting using electronic particle counters. The algae were grown in synthetic algal nutrient medium (SANM), (Miller et al., 1978).

Semi-continuous cultures were maintained in a Conviron Model E7H (Controlled Environments, Winnipeg, Canada) growth cabinet at 23 ± 2°C. Continuous light was supplied at 300 µmol quanta$\cdot m^{-2} \cdot s^{-1}$ by cool white fluorescent tubes and incandescent lamps arranged alongside and above cultures. Cells in the exponential growth phase (about 10^4 cells$\cdot m\ell^{-1}$) were used in the experiments. The exponential growth phase i.e. three days after inoculation, was determined from a graphical plot of daily cell counts, measured with an electronic particle counter, Coulter Multisizer II (Coulter Electronics, England).

The experimental set-up was the same as described by

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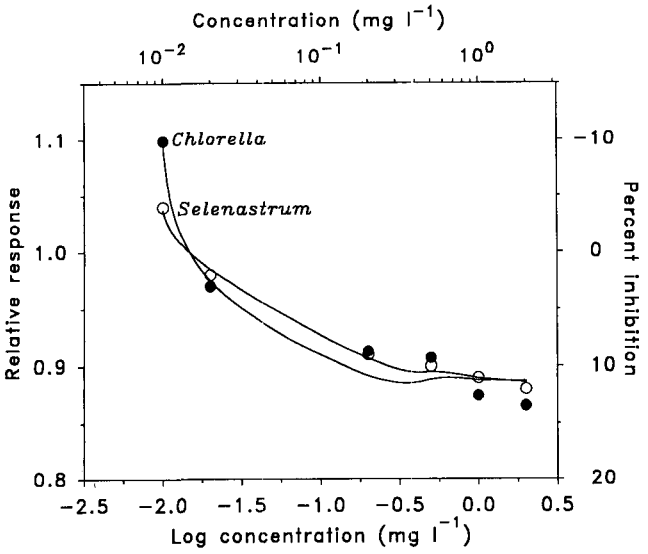

Figure 1
Dose-response curves of algae exposed to different concentrations of copper. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

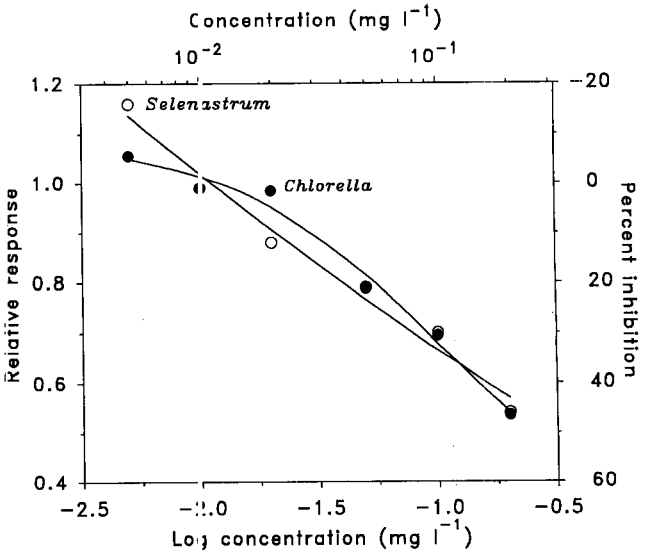

Figure 3
Dose-response curves of algae exposed to different concentrations of mercury. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

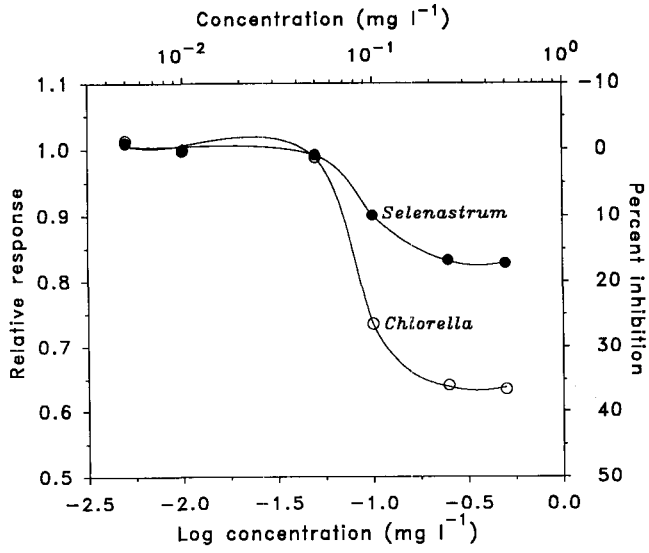

Figure 2
Dose-response curves of algae exposed to different concentrations of cadmium. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

Dubinsky et al. (1987). The O_2-chamber was irradiated from the front using an ordinary 35 mm slide projector. The measurements were all done at an irradiance equal to I_k, which is the irradiance at the onset of light saturation during photosynthesis.

I_k was calculated from P/I curves determined prior to the toxicity tests and varied between 250 to 320 µmol quanta·m^{-2}·s^{-1}. Different irradiancies (I) were brought about by selecting neutral density filters placed between the projector and the chamber. Irradiance measurements were taken with a Li-Cor Quantum Meter, Model LI-185B equipped with a LI-190SB quantum sensor placed at the rear of the chamber. The oxygen rates (as µg O_2·µg chl a^{-1}·min^{-1}) that were calculated from recorder traces were taken as (P) and plotted against the irradiancies and from these plots I_k was determined.

A single measurement at a specific irradiance is desirable for the purpose of toxicity tests because of the time constraints of most other tests. Following this approach, a sample could be tested in 30 min. I_k was chosen because this is the irradiance where a transition takes place from light limited to light saturated growth. We reasoned that any factor which could affect photosynthesis would be clearly seen at this irradiance level.

Algal samples were injected into the chamber through a hole at the top, that was closed with a pierced PVC screw cap during measurements. Toxic substances (Table 1) were injected, during incubation, through the 1 mm diameter capillary in the screw cap. Incubation time was 30 min for all treatments. The samples were mixed by means of a glass-covered magnetic spinbar driven by a magnetic stirrer. Oxygen was measured with a Clark-type electrode mounted in the side of the cuvet so that the membrane covered end just extended into the chamber. This system was calibrated once a day against distilled water air equilibrated at the experimental temperature, and against a zero oxygen solution (saturated Na_2SO_3 solution). The span in mV between zero oxygen (0% O_2) and air-saturated water (100% O_2) was measured at the experimental temperature and used in the calculations. These changes in O_2 concentration were recorded on a Graphic 1002 Y/t chart recorder (Lloyd Instruments, Southampton) and expressed as mV·min^{-1}. The mV·min^{-1} values were converted into µg O_2·t^{-1}·min^{-1} by means of calibration values and into µg O_2·µg chl a^{-1}·min^{-1} by dividing the oxygen rates with the chlorophyll *a* content (µg chl *a*·t^{-1}) and this value was then taken as the measured response for comparative purposes. The chlorophyll *a* content was determined and calculated by extracting chlorophyll *a* with 96% ethanol as described by Sartory and Grobbelaar (1984). The results were expressed as a response relative to the control

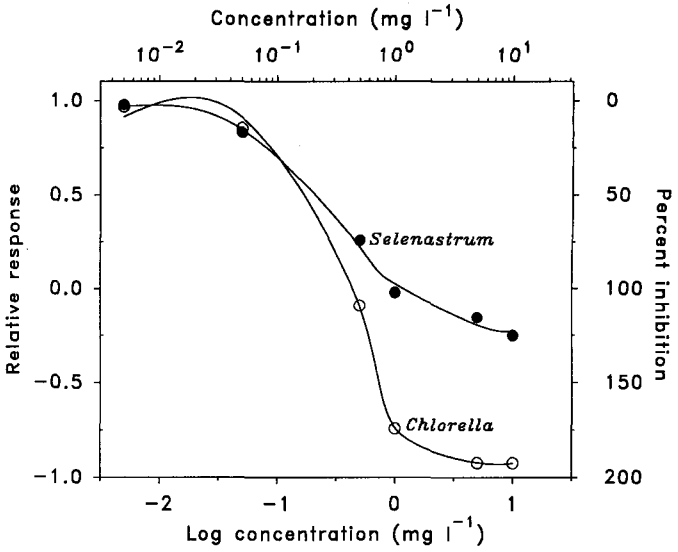

Figure 4
Dose-response curves of algae exposed to different concentrations of atrazine. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

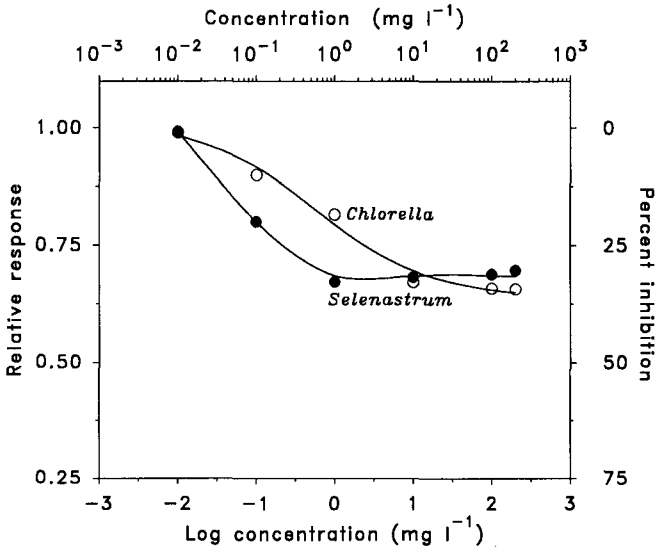

Figure 5
Dose-response curves of algae exposed to different concentrations of phenol. Oxygen-evolution was used as response variable and measured within 30 min of exposure to the toxin.

response, after calculation of dilution factors brought about by the addition of toxins directly to the sample. Dose-response curves were obtained from non-linear least square fits of the relative response values using a four-parameter logistic equation. EC-values were calculated from these non-linear fits by using the inverse of the regression curve according to Van der Heever and Grobbelaar (1996).

Results

An initial stimulative response in oxygen evolution rate (Fig. 1) of 10% and 5% (relative response of 1.1 and 1.05 respectively) was evident for *C. vulgaris* and for *S. capricornutum* which were measured at low concentrations (0.01 mg·ℓ^{-1}) respectively. After the initial stimulatory response a decline to 0.97 occurred with a doubling of copper concentration from 0.01 to 0.02 mg·ℓ^{-1}. A further tenfold increase in the copper concentration resulted in a relative response value of 0.92. At higher concentrations of 0.2 to 2 mg·ℓ^{-1} copper, there was only a 3% increase in response (0.90 to 0.87). The response of both species to copper was almost identical for the concentration range tested.

The effect of different concentrations of cadmium on oxygen evolution over an exposure period of 30 min to the heavy metal is shown in Fig. 2. No initial stimulation was seen at the low concentrations as was the case with copper dosages (Fig. 1). From an initial zero inhibition (1.0 relative response) at low concentrations ranging from 0.005 to 0.05 mg·ℓ^{-1}, *C. vulgaris* exhibited a sharp inhibition of almost 25% (relative response change of 0.25 from 1.0 to almost 0.75) when the concentration of cadmium was doubled to 0.1 mg·ℓ^{-1}. *Selenastrum capricornutum*, at this same concentration, only experienced a 10 % inhibition. With a two-and-a-half-fold increase in the cadmium concentration to 0.25 mg·ℓ^{-1}, the inhibition increased with a further 10% and 5% for *C. vulgaris* and for *S. capricornutum* respectively, after which the inhibition remained constant at a relative response of 0.65 (35% inhibition) and 0.85 (15% inhibition) respectively for *C. vulgaris* and for *S. capricornutum* at higher concentrations (0.25 and 0.5 mg·ℓ^{-1} cadmium).

Treatment of algal suspensions with mercury revealed that (Fig. 3) from an initial stimulation of 18% at low concentrations of 0.005 mg·ℓ^{-1} mercury for *S. capricornutum*, a linear relationship ($r^2 = 0.9895$) between log concentration and relative response was observed. An increase from 0.005 to 0.5 mg·ℓ^{-1} mercury resulted in a decrease in the relative response range of 1.2 to 0.5 (20% stimulation to 50% inhibition). *Chlorella vulgaris* responded in a similar fashion except for stimulations at low concentrations

TABLE 2
SUMMARY OF THE RESULTS FROM O_2-EVOLUTION TOXICITY EXPERIMENTS EXPRESSED AS EC VALUES IN mg·ℓ$^{-1}$

Compound	*S. capricornutum*			*C. vulgaris*		
	EC_{10}	EC_{50}	EC_{90}	EC_{10}	EC_{50}	EC_{90}
Copper	0.280	*nr	nr	0.147	nr	nr
Cadmium	0.100	nr	nr	0.072	nr	nr
Mercury	0.021	nr	nr	0.030	nr	nr
Atrazine	0.031	0.222	0.751	0.085	0.305	0.433
Gusathion	nr	nr	nr	nr	nr	nr
Phenol	0.076	nr	nr	0.143	nr	nr

*nr = no response

(0.005 to 0.2 mg·ℓ$^{-1}$ mercury).

Figure 4 shows an inverse sigmoidal dose-response curve for atrazine. This means that at low concentrations of 0.005 and 0.05 mg·ℓ$^{-1}$ the algae were unaffected. After this initial "no effects" phase there was an exponential inhibitory response which eventually ended in a constant phase at high concentrations (5 and 10 mg·ℓ$^{-1}$) where no further increase in inhibition was seen. The relative response of *S. capricornutum* ranged from 1 to 0 (i.e. 0% inhibition to 100% inhibition) whereas for *C. vulgaris* it ranged from 1 to almost -1 (i.e. 0 to 200% inhibition).

The organophosphate gusathion had no effect on the oxygen evolution. Shown in Fig. 5 is the effect which phenol had on the oxygen evolution of *S. capricornutum* and *C. vulgaris*. No inhibition was seen for *S. capricornutum* at a low concentration of 0.01 mg·ℓ$^{-1}$ whereafter it increased to a concentration of 1 mg·ℓ$^{-1}$. The inhibition remained constant at a relative response of 0.7 for concentrations of 1 to 200 mg·ℓ$^{-1}$ phenol. *Chlorella vulgaris* also exhibit an exponential decline (increase in inhibition) at low concentrations dosages, but the only difference is that this stabilised at 1 mg·ℓ$^{-1}$ and higher. The maximum inhibition of 30% was the same for both algae.

Discussion

The results clearly show that oxygen production, as measured in the oxygen electrode chamber, was affected by some of the compounds tested for their potential toxicity. EC_{50} and EC_{90} values could only be calculated for atrazine and mercury within the concentration ranges tested. From the EC_{10} values it can be seen that *S. capricornutum* is more sensitive to atrazine and phenol than *C. vulgaris* (Figs. 4 and 5; Table 2). *Chlorella vulgaris* on the other hand is more sensitive than *S. capricornutum* to copper and cadmium (Figs. 1 and 2; Table 2) at EC_{10} level.

The greater inhibition, as caused by Hg compared to Cu and Cd, can be ascribed to the interruption of electron flow by Hg at multiple sites such as plastocyanin (Katoh and Takamiya, 1964), the reaction centre of photosystem I (Kojima et al., 1987) and iron sulphur centres (Gelbeck et al., 1977). It is also known that the multiple effects of mercury arise from its interaction with the sulphydryl groups of protein (Passow et al., 1961). According to Shioi et al. (1978) copper directly inactivated the ferredoxin and reduced the $NADP^+$ photoreduction in the electron transport of photosynthesis. A secondary and less severe inhibitory effect of copper is in photosystem II (PSII) between the oxidising side of the reaction centre of PSII and the electron-donating site of DPC.

Rai et al. (1991) suggested, in a study on the toxicity of Hg and Zn to *C. vulgaris*, that Hg has a greater toxic potential than Zn. Its great penetration capacity into the thylakoid membrane, and multiple binding with the electron transport system coupled with ATP generation seems to be the major cause for the inhibition of electron transport, photophosphorylation and thus all biological activities. They also pointed out that PSII was the primary site of action of Hg in *C. vulgaris*. Hg, Cu and Cd impair the electron transport system because of impairment of ATP production, which is needed in the Calvin cycle. Thus overall cell metabolism is influenced and, therefore, O_2-production is affected. This chain of events would possibly apply to all eucaryotic algae.

At EC_{50} and EC_{90} levels the response of *S. capricornutum* and *C. vulgaris* to atrazine toxicity is contrary to the response as determined at the EC_{10} level (Table 2). *Chlorella vulgaris* is more sensitive than *S. capricornutum* to high atrazine concentrations, but *S. capricornutum* is more sensitive than *C. vulgaris* at the EC_{10} level. The drastic inhibition of photosynthesis substantiates the evidence that the Hill reaction (PSII) is inhibited by atrazine. The inhibition by atrazine is similar to that by DCMU according to Singh et al. (1983).

The chemical characteristics of the algal growth medium might be an important determining factor of toxicity thresholds. Turbak et al. (1986) reported an EC_{50} value from O_2-evolution assays of 69.7 µg·ℓ$^{-1}$ and when using natural water samples as growth medium an EC_{50} of 854 µg·ℓ$^{-1}$ was obtained for atrazine with *S. capricornutum* as test organism. Our results gave an EC_{50} of 222 µg·ℓ$^{-1}$ for *S. capricornutum* and 305 µg·ℓ$^{-1}$ for *C. vulgaris*. These results support observed variances obtained by different laboratories with identical test organisms and using different methods (Nyholm and Källqvist, 1989).

Gusathion had no effect on the photosynthetic rates of either test organisms. The reason for this is possibly that gusathion is an organophosphate and it is known that organophosphates could be utilised as phosphate source by algae. Phenols are toxic due to their oxidation to the corresponding quinones, which can readily oxidize sulphydryl groups on enzymes, thereby disrupting the normal oxidation-reduction balance in algae (Hanchey-Bauer, 1978). *Selenastrum capricornutum* was almost twice as sensitive towards phenol than was *C. vulgaris* as can be seen from Table 2. No related data could be found to compare our data with in order to compare the results of our method with those using other methods.

The major disadvantage of the oxygen-evolution method, as it was applied in our tests, is that it measures only one part (PSII activity) of one metabolic process, namely photosynthesis, rather than growth which can be considered the result of numerous metabolic processes. The primary advantage of the rapid O_2-measurements, however, is that it allows for the determination of photosynthetic rates using small samples (15 mℓ) and results could be obtained within 5 min, after the chamber and sample had been stabilised. For PSII system herbicides, such as atrazine, DCMU and many other, the O_2-evolution measurement is very practical.

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References

DUBINSKY Z, FALKOWSKI PG, POST AF and VAN HES UM (1987) A system for measuring phytoplankton photosynthesis in a defined light field with an oxygen electrode. *J. Plankton Res.* **9** 607-612.

GELBECK JH, LEIN S and SAN PIETRO A (1977) Isolation and characterization of a subchloroplast particle enriched in iron sulfur protein and P700. *Arch. Biochem. Biophys.* **178** 140-150.

HANCHEY-BAUER P (1978) Physiological plant pathology. In: Salisbury FB and Ross CW (eds.) *Plant physiology* (2nd edn). Wadsworth Publishing Company, Belmont, California. 399-402.

HOSTETTER HP (1976) A rapid bioassay for algal nutrients and toxins. *J. Phycol.* **12** (Suppl) 10.

KATOH S and TAKAMIYA A (1964) Nature of copper binding in spinach plastocyanin. *J. Biochem.* **55** 378-387.

KOJIMA Y, HIYAMA T and SAKURAI H (1987) Effects of mercurials on iron sulphur centres of PSI of *Anacystis nidulans*. In: Biggins J (ed.) *Progress in Photosynthesis Research*. Nijhoff/Junk, The Hague. 57-60.

MILLER WE, GREENE JC and SHIROYAMA T (1978) The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA 660/9-78-018, US Environmental Protection Agency, Corvallis, OR

NYHOLM N and KÄLLQVIST T (1989) Methods for growth inhibition toxicity tests with freshwater algae. *Environ. Toxicol. Chem.* **8** 689-703.

PASSOW H, ROTHSTEIN A and CLARKSON TW (1961) The general pharmacology of heavy metals. *Pharmacol. Rev.* **13** 185-224.

RAI LC, SINGH AK and MALLICK N (1991) Studies on photosynthesis, the associated electron transport system and some physiological variables of *Chlorella vulgaris* under heavy metal stress. *J. Plant Physiol.* **137** 419-424.

SARTORY DP and GROBBELAAR JU (1984) Extraction of chlorophyll *a* from freshwater phytoplankton for spectrophotometric analysis. *Hydrobiol.* **114** 177-187.

SHIOI Y, HIROKO T and TSUTOMU S (1978) Effects of copper on photosynthesis electron transport systems in spinach chloroplasts. *Plant Cell Physiol.* **19** 203-209.

SINGH RK, SINGH BD and SINGH HN (1983) Inhibition of photosystem II of nitrogen-fixing blue-green alga *Nostoc linckia* by the rice-field herbicide benthiocarb. *Z. Allg. Mikrobiol.* **23** 435-441.

TURBAK SC, OLSON SB and McFETERS GA (1986) Comparison of algal assay systems for detecting waterborne herbicides and metals. *Water Res.* **20** 91-96.

VAN DER HEEVER JA and GROBBELAAR JU (1996) The use of *Selenastrum capricornutum* growth potential as a measure of toxicity of a few selected compounds. *Water SA* **22** 183 -191.